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jc503 U.S. PRO

Attorney Docket No.: 5709.200-U.S.

PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FILING UNDER 37 C.F.R. 1.53(b)

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

Express Mail Label No. EL021372095US
Date of Deposit November 16, 1999

Sir:

jc503 U.S. PRO
09/441313
11/16/99

This is a request for filing a patent application under 37 C.F.R. 1.53(b) of
Applicant(s): Svendsen et al.

Title: α -amylase variants

46 pages of specification 4 sheets of drawings

4 sheets of Declaration and Power of Attorney

1 page of abstract 24 pages of sequence listing

[x] The filing fee is calculated as follows:

Basic Fee: \$760.00

Total Claims: $23 - 20 = 3 \times 18 =$ \$ 54.00

Independent Claims: $3 - 3 = 0 \times 78 =$ \$ 0

Total Fee: \$814.00

The benefit of application no. 09/193,068 filed on November 16, 1998 in the U.S.
is claimed under 35 U.S.C. 120.

Address all future communications to Steve T. Zelson, Esq., Novo Nordisk of
North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174-6401.

Please charge the required fee, estimated to be \$814, to Novo Nordisk of North America, Inc., Deposit Account No. 14-1447. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: November 16, 1999



Elias J. Lambiris, Reg. No. 33,728
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Attorney Docket No.: 5709.200-U.S.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

Re: U.S. Patent Application for
Title: α -amylase variants
Applicants: Svendsen et al.

Sir:

Express Mail Label No. EL021372095US

Date of Deposit : November 16, 1999

I hereby certify that the following attached paper(s) or fee

1. Filing Under 37 C.F.R. 1.53(b) (in duplicate)
2. Patent Application
3. Unexecuted Combined Declaration and Power of Attorney
4. Preliminary Amendment

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, DC 20231.

Carol A. McFarlane

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Svendsen et al.

Application No.: To be assigned

Group Art Unit: To be assigned

Filed: November 16, 1999

Examiner: To be assigned

For: α -amylase variants

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Before the above-captioned application is taken up for examination, entry of the following amendment is respectfully requested:

IN THE SPECIFICATION:

At page 1, after the title, insert

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial no. 09/193,068 filed on November 16, 1998, the contents of which are fully incorporated herein by reference.--

IN THE CLAIMS:

Please cancel claims 19-23, 25-26, 28, 30, 32-40 without prejudice or disclaimer.

Please amend claim 5 as follows:

At lines 2 and 3, delete "as defined in any of claims 1-3".

Please amend claim 6 as follows:

At line 1, delete "of any of claims 1-5" and insert --according to claim 1--.

Please amend claim 7 as follows:

At line 1, delete "accordin" and insert --according--.

Please amend claim 8 as follows:

At line 1, delete "claims 1-6" and insert --claim 1--.

Please amend claim 9 as follows:

At line 1, delete "any of claims 1-8" and insert --claim 1--.

Please amend claim 10 as follows:

At line 1, delete "any of claims 1-10" and insert --claim 1--.

Please amend claim 11 as follows:

At line 1, delete "accordint to claims 1-10" and insert --according to claim 1--.

Please amend claim 13 as follows:

At line 1, delete "any of claims 1-12" and insert --claim 1--.

Please amend claim 16 as follows:

At line 1, delete "claims 1 to 15" and insert --claim 1--.

Please amend claim 17 as follows:

At line 1, delete "any of claims 1 to 16" and insert --claim 1--.

Please amend claim 18 as follows:

At line 2, delete "any one of claims 1 to 17" and insert --claim 1--.

Please amend claim 24 as follows:

At line 2, delete "any one of claims 1 to 17" and insert --claim 1--.

Please amend claim 27 as follows:

At line 2, delete "any of claims 1 to 17" and insert --claim 1--.

Please amend claim 29 as follows:

At lines 2 and 3, delete "any of claims 1 to 17" and insert --claim 1--.

Please amend claim 31 as follows:

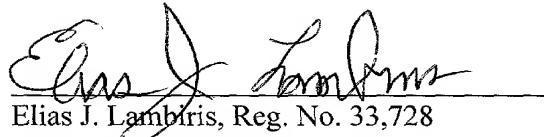
At line 2, delete "any of claims 1 to 17" and insert --claim 1--.

REMARKS

This amendment is submitted to reduce filing fees and to correct improper multiple dependent claims. Since only dependencies are altered, there is no new matter added, and entry of the amendment is respectfully requested.

Respectfully submitted,

Date: November 16, 1999


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Title: α -amylase variants

FIELD OF THE INVENTION

The present invention relates to novel variants of parent s Termamyl-like α -amylases with altered properties relative of the parent alpha-amylase. Said properties include increased stability, e.g., at acidic pH, e.g., at low calcium concentrations and/or high temperatures. Such variants are suitable for a number of applications, in particular, industrial 10 starch processing (e.g., starch liquefaction or saccharification).

BACKGROUND OF THE INVENTION

α -Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) 15 constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of 20 enzymes. A number of α -amylase such as Termamyl-like α -amylases variants are known from, e.g., WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

WO 96/23874 provides the three-dimensional, X-ray crystal structural data for a Termamyl-like α -amylase which consists of 25 the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* α -amylase and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α -amylase comprising the amino acid sequence (the latter being available commercially under the tradename TermamylTM), and which is thus closely 30 related to the industrially important *Bacillus* α -amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α -amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* α -amylases). WO 96/23874 further describes 35 methodology for designing, on the basis of an analysis of the

structure of a parent Termamyl-like α -amylase, variants of the parent Termamyl-like α -amylase which exhibit altered properties relative to the parent.

5 **BRIEF DISCLOSURE OF THE INVENTION**

The present invention relates to novel α -amylolytic variants (mutants) of a Termamyl-like α -amylase, in particular variants exhibiting increased stability at acidic pH at high temperatures (relative to the parent) which are advantageous in 10 connection with, e.g., the industrial processing of starch (starch liquefaction, saccharification and the like) as described in US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

15 **Starch conversion**

A "traditional" starch conversion process degrading starch to lower molecular weight carbohydrate components such as sugars or fat replacers includes a debranching step.

20 **"Starch to sugar" conversion**

In the case of converting starch into a sugar the starch is depolymerized. A such depolymerization process consists of a pretreatment step and two or three consecutive process steps, viz. a liquefaction process, a saccharification process and 25 dependent on the desired end product optionally an isomerization process.

Pre-treatment of native starch

Native starch consists of microscopic granules which are 30 insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typically 35 industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today

mostly obtained by enzymatic degradation.

Liquefaction

During the liquefaction step, the long chained starch is degraded into branched and linear shorter units (maltodextrins) by an α -amylase (e.g., Termamyl™ SEQ ID NO: 4 herein). The liquefaction process is carried out at 105-110°C for 5 to 10 minutes followed by 1-2 hours at 95°C. The pH lies between 5.5 and 6.2. In order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

Saccharification

After the liquefaction process the maltodextrins are converted into dextrose by addition of a glucoamylase (e.g., AMG™) and a debranching enzyme, such as an isoamylase (US Patent 4,335,208) or a pullulanase (e.g., Promozyme™) (US Patent 4,560,651). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C) to inactivate the liquefying α -amylase to reduce the formation of short oligosaccharide called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

Normally, when denaturing the α -amylase after the liquefaction step about 0.2-0.5% of the saccharification product is the branched trisaccharide α -glucosyl maltose (panose) which cannot be degraded by a pullulanase. If active amylase from the liquefaction step is present during saccharification (i.e., no denaturing), this level can be as high as 1-2%, which is highly undesirable as it lowers the saccharification yield significantly.

Isomerization

When the desired final sugar product is e.g. high fructose syrup the dextrose syrup may be converted into fructose.

5 After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucoseisomerase (such as Sweetzyme™).

10

In the context of the invention the term "acidic pH" means a pH below 7.0, especially below the pH range in which industrial starch liquefaction processes are traditionally performed, as described above, which is between pH 5.5 and 6.2.

15

In the context of the present invention the term "low Calcium concentration" means concentrations below the normal level used in traditional industrial starch liquefaction processes, such as between 0-40 ppm, preferably between 10-30 ppm, such as between 15-25 ppm Calcium. Normal concentrations vary depending of the concentration of free Ca^{2+} in the corn. Normally a dosage corresponding to 1mM (40ppm) is added which together with the level in corn gives between 40 and 60 ppm free Ca^{2+} .

20

In the context of the invention the term "high temperature" means temperatures between 95 and 160°C, especially the temperature range in which industrial starch liquefaction processes are normally performed, which is between 95 and 105°C.

25

The invention further relates to DNA constructs encoding variants of the invention, to methods for preparing variants of the invention, and to the use of variants of the invention, alone or in combination with other α -amylolytic enzymes, in various industrial processes, in particular starch liquefaction.

30 35 Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used.

For ease of reference, α -amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or 15 Δ (A30-N33).

Where a specific α -amylase contains a "deletion" in comparison with other α -amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively. Multiple mutation may also be separated as follows, i.e., meaning the same as the plus sign:

Ala30Asp/Glu34Ser or A30N/E34S

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

30 A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue 35 may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine

in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

5

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α -amylases in the context of the invention. The numbers on the Extreme left designate the respective amino acid sequences as follows:

- 1: SEQ ID NO: 2,
- 2: amylase
- 3: SEQ ID NO: 1,
- 4: SEQ ID NO: 5,
- 15 5: SEQ ID NO: 4,
- 6: SEQ ID NO: 3.

Figure 2 shows the PCR strategy used in Example 1.

20 DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α -amylase

It is well known that a number of α -amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 4 (commercially available as TermamylTM) has been found to be about 89% homologous with the *B. amyloliquefaciens* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 5 and about 79% homologous with the *B. stearothermophilus* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α -amylases include an α -amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α -amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

Still further homologous α -amylases include the α -amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α -amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* ⁵ α -amylases are OptithermTM and TakathermTM (available from Solvay), MaxamylTM (available from Gist-brocades/Genencor), Spezym AATM and Spezyme Delta AATM (available from Genencor), and KeistaseTM (available from Daiwa).

Because of the substantial homology found between these α -¹⁰ amylases, they are considered to belong to the same class of α -amylases, namely the class of "Termamyl-like α -amylases".

Accordingly, in the present context, the term "Termamyl-like α -amylase" is intended to indicate an α -amylase which, at the amino acid level, exhibits a substantial homology to TermamylTM, ¹⁵ i.e., the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4 herein. In other words, a Termamyl-like α -amylase is an α -amylase which has the amino acid sequence shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 of WO ²⁰ 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) or i) which displays at least 60% homology ²⁵ (identity), preferred at least 70%, more preferred at least 75%, even more preferred at least 80%, especially at least 85%, especially preferred at least 90%, especially at least 95%, even especially more preferred at least 97%, especially at least 99% homology with at least one of said amino acid sequences shown in ³⁰ SEQ ID NOS 1: or 2 or 3 or 4 or 5 or 6 or 7 or 8 and/or ii) displays immunological cross-reactivity with an antibody raised against one or more of said α -amylases, and/or iii) is encoded by a DNA sequence which hybridizes, under the low to very high stringency conditions (said conditions described below) to the

DNA sequences encoding the above-specified α -amylases which are apparent from SEQ ID NOS: 9, 10, 11, 12, and 32, respectively, of the present application (which encodes the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4, and 5 herein, 5 respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

In connection with property i), the "homology" (identity) may be determined by use of any conventional algorithm, preferably by use of the gap programme from the GCG package version 8 (August 1994) using default values for gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 15 0.1 (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 8, 575 Science Drive, Madison, Wisconsin, USA 53711).

The parent Termamyl-like α -amylase backbone may in an embodiment have an amino acid sequence which has a degree of 20 identity to SEQ ID NO: 4 of at least 65%, preferably at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 97%, and even more preferably at least 99% identity 25 determined as described above

A structural alignment between Termamyl® (SEQ ID NO: 4) and a Termamyl-like α -amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α -amylases. One method of obtaining said structural alignment is 30 to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading 35 (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998).

For example, the corresponding positions, of target residues found in the C-domain of the *B. licheniformis* α -amylase, in the amino acid sequences of a number of Termamyl-like α -amylases which have already been mentioned are as follows:

5 _____

Termamyl-like α -amylase

10 <i>B. lich.</i> (SEQ ID NO: 4)	S356 Y358 E376 S417 A420
<i>B. amylo.</i> (SEQ ID NO: 5)	S356 Y358 E376 S417 A420
<i>B. stearo.</i> (SEQ ID NO: 3)	---- Y361 ---- ---- ----
<i>Bac.WO 95/26397</i> (SEQ ID NO: 2)	---- Y363 ---- S419 ----
<i>Bac.WO 95/26397</i> (SEQ ID NO: 1)	---- Y363 ---- ---- ----

15 _____

As will be described further below mutations of these conserved amino acid residues are very important in relation to increasing the stability at acidic pH and/or at low calcium concentration at high temperatures.

Property ii) (see above) of the α -amylase, i.e., the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α -amylase. The antibody, which may either 25 be monoclonal or polyclonal, may be produced by methods known in the art, e.g., as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g., as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α -amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8 respectively, 30 have been found.

35 The oligonucleotide probe used in the characterization of the Termamyl-like α -amylase in accordance with property iii)

above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α -amylase in question.

Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100mM ATP for 18 hours at ~40°C, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at -75°C (very high stringency). More details about the hybridization method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an α -amylase produced or producible by a strain of the organism in question, but also an α -amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α -amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α -amylase in question. The term is also intended to indicate that the parent α -amylase may be a variant of a naturally occurring α -amylase, i.e., a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α -amylase.

30 Parent hybrid α -amylases

The parent α -amylase (backbone) may be a hybrid α -amylase, i.e., an α -amylase which comprises a combination of partial amino acid sequences derived from at least two α -amylases.

The parent hybrid α -amylase may be one which on the basis of

amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α -amylase family. In this case, the hybrid α -amylase is typically composed of at least one part of a Termamyl-like α -amylase and part(s) of one or more other α -amylases selected from Termamyl-like α -amylases or non-Termamyl-like α -amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α -amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like α -amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α -amylase, or from at least one Termamyl-like and at least one fungal α -amylase. The Termamyl-like α -amylase from which a partial amino acid sequence derives may, e.g., be any of those specific Termamyl-like α -amylase referred to herein.

For instance, the parent α -amylase may comprise a C-terminal part of an α -amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α -amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α -amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α -amylase. A such hybrid Termamyl-like α -amylase may be identical to the *Bacillus licheniformis* α -amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) is replaced with the N-terminal 33 amino acid residues of the mature protein of the *Bacillus amyloliquefaciens* α -amylase (BAN) shown in SEQ ID NO: 5. A such hybrid may also consist of an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α -amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α -amylase

having the amino acid sequence shown in SEQ ID NO: 4.

The non-Termamyl-like α -amylase may, e.g., be a fungal α -amylase, a mammalian or a plant α -amylase or a bacterial α -amylase (different from a Termamyl-like α -amylase). Specific examples of such α -amylases include the *Aspergillus oryzae* TAKA α -amylase, the *A. niger* acid α -amylase, the *Bacillus subtilis* α -amylase, the porcine pancreatic α -amylase and a barley α -amylase. All of these α -amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α -amylase as referred to herein.

The fungal α -amylases mentioned above, i.e. derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α -amylases. The fungal α -amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

Furthermore, when a particular variant of a Termamyl-like α -amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g., deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α -amylase, it is to be understood that variants of another Termamyl-like α -amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

A preferred embodiment of a variant of the invention is one derived from a *B. licheniformis* α -amylase (as parent Termamyl-like α -amylase), e.g., one of those referred to above, such as the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

30

Altered properties of variants of the invention

The following discusses the relationship between alterations/mutations which may be present in variants of the invention, and desirable alterations in properties (relative to

those a parent, Termamyl-like α -amylase) which may result therefrom.

Increased stability at acidic pH and/or low calcium concentration at high temperatures

The present invention relates to a variant of a parent Termamyl-like α -amylase, which variant α -amylase has been altered in comparison to the parent α -amylase in one or more solvent exposed amino acid residues on the surface of the α -amylase to increase the overall hydrophobicity of the α -amylase and/or to increase the overall numbers of methyl groups in the sidechains of said solvent exposed amino acid residues on the surface.

In a preferred embodiment one or more solvent exposed amino acid residues on a concav surface with inwards bend are altered to more hydrophobic amino acid residues.

In another preferred embodiment one or more solvent exposed amino acid residues on a convex surface are altered to increase the number of methyl groups in the sidechain.

The present invention relates to an α -amylase variant of a parent Termamyl-like α -amylase, comprising an alteration at one or more positions selected from the group of:

E376, S417, A420, S356, Y358;

wherein (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid,

(b) the variant has α -amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like α -amylase having the amino acid sequence of SEQ ID NO: 4.

In an embodiment the alteration is one of the following

substitutions:

E376A, R, D, C, Q, G, H, I, K, L, M, N, F, P, S, T, W, Y, V.

In a preferred embodiment the substitution is: E376K.

In an embodiment the alteration is one of the following

substitutions: S417A, R, D, C, E, Q, G, H, I, K, L, M, N, F, P, T, W, Y, V;

In a preferred embodiment the substitution is S417T.

In an embodiment the alteration is one of the following substitutions A420R, D, C, E, Q, G, H, I, K, L, M, N, F, P, S, T, W, Y, V;

In a preferred embodiment the substitution is: A420Q, R.

10 In an embodiment the alteration is one of the following substitutions: S356A, R, D, C, E, Q, G, H, I, K, L, M, N, F, P, T, W, Y, V.

In an embodiment the alteration is one of the following substitutions Y358A, R, D, C, E, Q, G, H, I, K, L, M, N, F, P, S, T, W, V.

In a preferred embodiment the substitution is Y358F.

15 In an embodiment of the invention a variant comprises one or more of the following substitutions: E376K, S417T, A420Q, R, S356A, Y358F.

The increase in stability at acidic pH and/or low calcium concentration at high temperatures may be determined using the 20 method described below in Example 2 illustrating the invention.

The parent Termamyl-like α -amylase used as the backbone for preparing variants of the invention may be any Termamyl-like α -amylases as defined above.

Specifically contemplated are parent Termamyl-like α -amylases selected from the group derived from *B. licheniformis*, 25 such as *B. licheniformis* strain ATCC 27811, *B. amyloliquefaciens*, *B. stearothermophilus*, *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, and the parent Termamyl-like α -amylases depicted in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8.

30 In an embodiment of the invention the parent Termamyl-like α -amylase is a hybrid α -amylase being identical to the *Bacillus licheniformis* α -amylase shown in SEQ ID NO: 4 (Termamyl), except that the N-terminal 35 amino acid residues (of the mature protein) is replaced with the N-terminal 33 amino acid residues 35 of the mature protein of the *Bacillus amyloliquefaciens* α -amylase (BAN) shown in SEQ ID NO: 5. The parent Termamyl-like

hybrid α -amylase may be the above mentioned hybrid Termamyl-like α -amylase which further has the following mutations: H156Y+181T+190F+209V+264S (using the numbering in SEQ ID NO: 4). Said backbone is referred to below as "LE174".

- 5 The parent α -amylase may advantageously further have a mutation in one or more of the following positions: K176, I201 and H205 (using the numbering in SEQ ID NO: 4), especially one or more the following substitutions: K176R, I201F, and H205N (using the numbering in SEQ ID NO: 4), such as specifically the
10 following substitutions: K176R+I201F+H205N (using the numbering in SEQ ID NO: 4).

The inventors have found that the above mentioned variants have increased stability at pHs below 7.0 (i.e., acidic pH) and/or at calcium concentration below 1mM (40ppm) (i.e., low 15 calcium concentrations) at temperatures in the range from 95 to 160°C (i.e., high temperatures) relative to the parent Termamyl-like α -amylase.

Alterations (e.g., by substitution) of one or more solvent exposed amino acid residues which 1) increase the overall 20 hydrophobicity of the enzyme, or 2) increase the number of methyl groups in the sidechains of the solvent exposed amino acid residues improve the temperature stability. It is preferred to alter (e.g., by substitution) to more hydrophobic residues on a concav surface with inwards bend. On a convex surface, 25 alterations (e.g., by substitution) to amino acid residues with an increased number of methyl groups in the sidechain are preferred.

Using the program CAST found on the internet at <http://sunrise.cbs.umn.edu/cast/> version 1.0 (release Feb. 30 1998), (reference: Jie Liang, Herbert Edelsbrunner, and Clare Woodward. 1998. Anatomy of protein Pockets and Cavities: Measurements of binding site geometry and implications for ligand design. Protein Science, 7, pp. 1884-1897), a concave area which access to the surface can be identified. Access to 35 the surface is in the program defined as a probe with a diameter of 1.4 \AA can pass in and out. Using default parameters in the

CAST program concave cavities can be found using the Calcium depleted alpha-amylase structure from *B. licheniformis* as found in the Brookhaven database (1BPL):

Three types of interaction can be rationalised:

- 5 A. Interaction between the sidechain of the residue and the protein,
 - B. Interaction between the sidechain of the residue and the surrounding water,
 - C. Interaction between the water and the protein.
- 10 Using the parent Termamyl-like α -amylase shown in SEQ ID NO: 4 as the backbone the following positions are considered to be solvent exposed and may suitably be altered:
E376, S417, A420, S356, Y358.

Corresponding and other solvent exposed positions on the 15 surface of other Termamyl-like α -amylase may be identified using the dssp program by W. Kabsch and C. Sander, Biopolymers 22 (1983) pp. 2577-2637. The convex surfaces can be identified using the AACAVI program part from the WHATIF package (G. Vriend, Whatif and drug design program. J. Mol. Graph. 8, pp. 20 52-56. (1990) version 19980317).

In an embodiment of the invention a variant comprises one or more of the following substitutions: E376K, S417T, A420Q,R, S356A, Y358F.

The inventors have found that the stability at acidic pH 25 and/or low calcium concentration at high temperatures may be increased even more by combining mutations in the above mentioned positions, i.e., E376, S417, A420, S356, Y358, (using the SEQ ID NO: 4 numbering) with mutations in one or more of positions K176, I201, and H205.

30 The following additional substitutions are preferred:

K176A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V;
I201A,R,D,C,E,Q,G,H,L,K,M,N,F,P,S,T,W,Y,V;
H205A,R,D,C,E,Q,G,I,L,K,M,N,F,P,S,T,W,Y,V;

As also shown in Example 2 illustrating the invention 35 combining the following mutations give increased stability:
K176+I201F+H205N+E376K+A420R or

K176+I201F+H205N+S417T+A420Q or

K176+I201F+H205N+S356A+Y358F using the hybrid α -amylase referred to as LE174 as the parent Termamyl-like α -amylase.

5 General mutations in variants of the invention

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues present in the part of the α -amylase variant 10 which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

Analogously, it may be preferred that one or more cysteine 15 residues present among the amino acid residues with which the parent α -amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or leucine.

Furthermore, a variant of the invention may - either as the 20 only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the 25 replacement, in the Termamyl-like α -amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses variants incorporating two or more of the above outlined 30 modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

Cloning a DNA sequence encoding an α-amylase of the invention

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific

primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α -amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α -amylase-encoding sequence, is created in a vector carrying the α -amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into α -amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in s question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent α -amylase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present 10 invention relates to a method for generating a variant of a parent α -amylase, e.g., wherein the variant exhibits altered or increased thermal stability relative to the parent, the method comprising:

- (a) subjecting a DNA sequence encoding the parent α -amylose to random mutagenesis,
- (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
- (c) screening for host cells expressing an α -amylase variant which has an altered property (i.e. thermal stability) 20 relative to the parent α -amylase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of 25 a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, 30 and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the alpha-amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α -amylase is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the α -amylase by, e.g., transforming a plasmid containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent alpha-amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus latus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram-negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

20 Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent α -amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

Alternative methods of providing α-amylase variants

Alternative methods for providing variants of the invention include gene shuffling method known in the art including the methods, e.g., described in WO 95/22625 (from Affymax Technologies N.V.) and WO 96/00343 (from Novo Nordisk A/S).

Expression of α-amylase variants of the invention

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the lac operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA*

promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α -amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α -amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced

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by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α -amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α -amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lenthus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or grammegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α -amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α -amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

The α -amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

30 Industrial Applications

The α -amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. An enzyme variant of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning detergent compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for

textile desizing. Conditions for conventional starch- conversion processes, including starch liquefaction and/or saccharification processes, are described in, e.g., US 3,912,590 and in EP patent publications Nos. 252,730 and 63,909.

5

Production of sweeteners from starch:

A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes, viz. a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to dextrans by an α -amylase (e.g. TermamylTM) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2 hours. In order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrans are converted into dextrose by addition of a glucoamylase (e.g. AMGTM) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g. PromozymeTM). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C), and the liquefying α -amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucoseisomerase (such as SweetzymeTM).

At least 1 enzymatic improvements of this process could be envisaged. Reduction of the calcium dependency of the liquefying α -amylase. Addition of free calcium is required to ensure adequately high stability of the α -amylase, but free calcium strongly inhibits the activity of the glucoseisomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium to below 3-5 ppm. Cost savings could be obtained if such an

operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α -amylase which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α -amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α -amylase.

α -amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α -amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

MATERIALS AND METHODS

30 Enzymes:

LE174 hybrid alpha-amylase variant: LE174 is a hybrid Termamyl-like alpha-amylase being identical to the Termamyl sequence, i.e., the *Bacillus licheniformis* α -amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the *Bacillus amyloliquefaciens*

alpha-amylase shown in SEQ ID NO: 5, which further have following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

5 Construction of pSNK101

This *E. coli/Bacillus* shuttle vector can be used to introduce mutations without expression of α -amylase in *E. coli* and then be modified in such way that the α -amylase is active in *Bacillus*. The vector was constructed as follows: The α -amylase gene in the pX vector (pDN1528 with the following alterations within amyL: BAN(1-33), H156Y, A181T, N190F, A209V, Q264S, the plasmid pDN1528 is further described in Example 1) was inactivated by interruption in the *PstI* site in the 5' coding region of the alpha-amylase gene by a 1.2 kb fragment containing an *E. coli* origin fragment. This fragment was amplified from the pUC19 (GenBank Accession #: X02514) using the forward primer 1: 5'-gacctgcagtcaggcaacta-3' (SEQ ID NO: 28) and the reverse primer 1: 5'-tagagtcgacotgcaggcat-3' (SEQ ID NO: 29). The PCR amplicon and the pX plasmid containing the α -amylase gene were digested with *PstI* at 37°C for 2 hours. The pX vector fragment and the *E. coli* origin amplicon were ligated at room temperature, for 1 hour and transformed in *E. coli* by electrotransformation. The resulting vector is designated pSNK101.

This *E. coli/Bacillus* shuttle vector can be used to introduce mutations without expression of α -amylase in *E. coli* and then be modified in such way that the α -amylase is active in *Bacillus*. The vector was constructed as follows: The α -amylase gene in the pX vector (pDN1528 with the following alterations within amyL: BAN(1-33), H156Y+A181T+N190F+A209V+Q264S, the plasmid pDN1528 is further described in Example 1) was inactivated by interruption in the *PstI* site in the 5' coding region of the alpha-amylase gene by a 1.2 kb fragment containing an *E. coli* origin fragment. This fragment was amplified from the pUC19 (GenBank Accession

#:X02514) using the forward primer 2: 5'-gacctgcagtcaggcaacta-
3' (SEQ ID NO: 30) and the reverse primer 2: 5'-tagagtcgacacctgcaggcat-3' (SEQ ID NO: 31). The PCR amplicon and
the pX plasmid containing the α -amylase gene were digested
with PstI at 37°C for 2 hours. The pX vector fragment and the
E. coli origin amplicon were ligated at room temperature for 1
hour and transformed in *E. coli* by electrotransformation. The
resulting vector is designated pSnK101.

10 **Low pH filter assay**

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 μ g/ml chloramphenicol at 37°C for at least 21 hrs. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 90°C for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on assay plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours. at 50°C. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Secondary screening

Positive transformants after rescreening are picked from the storage plate and tested in a secondary plate assay.

Positive transformants are grown for 22 hours at 37°C in 5 ml LB + chloramphenicol. The *Bacillus* culture of each positive transformant and a control LE174 variant were incubated in citrate buffer, pH 4.5 at 90°C and samples were taken at 5 0,10,20,30,40,60 and 80 minutes. A 3 microliter sample was spotted on a assay plate. The assay plate was stained with 10% Lugol solution. Improved variants were seen as variants with higher residual activity detected as halos on the assay plate than the backbone. The improved variants are determined by 10 nucleotide sequencing.

Fermentation and purification of α -amylase variants

A *B. subtilis* strain harbouring the relevant expression plasmid is streaked on a LB-agar plate with 15 μ g/ml chloramphenicol from -80°C stock, and grown overnight at 37°C. The colonies are transferred to 100 ml BPX media supplemented with 15 μ g/ml chloramphenicol in a 500 ml shaking flask.

Composition of BPX medium:

Potato starch	100	g/l
Barley flour	50	g/l
BAN 5000 SKB	0.1	g/l
Sodium caseinate	10	g/l
Soy Bean Meal	20	g/l
Na ₂ HPO ₄ , 12 H ₂ O	9	g/l
Pluronic™	0.1	g/l

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear 30 solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 35 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions

which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

5

Stability determination

All the stability trials are made using the same set up. The method is:

The enzyme is incubated under the relevant conditions (1-
10 4). Samples are taken at 0, 5, 10, 15 and 30 minutes and
diluted 25 times (same dilution for all taken samples) in assay
buffer (0.1M 50mM Britton buffer pH 7.3) and the activity is
measured using the Phadebas assay (Pharmacia) under standard
conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) is used
as reference (100%). The decline in percent is calculated as a
function of the incubation time. The table shows the residual
activity after 30 minutes of incubation.

20 Activity determination - (KNU)

One Kilo alpha-amylase Unit (1 KNU) is the amount of enzyme
which breaks down 5.26 g starch (Merck, Amylum Solubile, Erg., B
6, Batch 9947275) per hour in Novo Nordisk's standard method for
determination of alpha-amylase based upon the following
25 condition:

Substrate	soluble starch
Calcium content in solvent	0.0043 M
Reaction time	7-20 minutes
Temperature	37°C
pH	5.6

Detailed description of Novo Nordisk's analytical method (AF 9)
is available on request.

Specific activity determination

35 Assay for α -Amylase Activity

α -amylase activity is determined by a method
employing Phadebas® tablets as substrate. Phadebas tablets

(Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl₂, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The α -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α -amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α -amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α -amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α -amylase protein) of the α -amylase in question under the given set of conditions.

30 EXAMPLES

Example 1.

Construction, by random mutagenesis, of Termamyl-like LE174 α -amylase variants having an improved stability at low pH and a reduced dependency on calcium ions for stability compared to the parent enzyme.

5

Random mutagenesis

To improve the stability at low pH and low calcium concentration of the parent LE174 α -amylase variant random mutagenesis in preselected regions was performed.

10 The regions were:

Region: Residue:

SERI A425-Y438

SERII W411-L424

SERIII G397-G410

SERV T369-H382

SERVII G310-F323

SERIX L346-P359

For each six region, random oligonucleotides are synthesized using the same mutation rate (97 % backbone and 1% of each of the three remaining nucleotides giving 3% mutations) in each nucleotide position in the above regions, e.g., 1. position in codon for A425: 97%C, 1%A, 1%T, 1%G. The six random oligonucleotides and if used complementary SOE helping primers are shown in tables1-6: with the four distribution of 25 nucleotides below.

Table 1.

RSERI: 5'-GC GTT TTG CCG GCC GAC ATA 312 234 322 243 333 133
444 233 423 242 212 211 243 343 CAA ACC TGA ATT-3' (SEQ ID NO:
30 15)

Table 2.

RSERII: 5'-GC GTT TTG CCG GCC GAC ATA CAT TCG CTT TGC CCC ACC
GGG TCC GTC TGT TAT TAA TGC CGC 311 133 241 122 243 113 341 432
35 423 433 223 332 242 331 GCC GAC AAT GTC ATG GTG-3' (SEQ ID NO:
16)

Table 3.

RSERIII: 5'-GTC GCC TTC CCT TGT CCA 433 413 112 423 124 424 423
 411 121 123 124 324 243 233 GTA CGC ATA CTG TTT TCT-3' (SEQ ID
 NO: 17)

Helping primer FSERIII: 5'-TGG ACA AGG GAA GGC GAC AG-3' (SEQ ID NO: 18)

Table 4.

10 RSERV: 5-TAA GAT CGG TTC AAT TTT 424 222 311 443 144 112 223
 434 324 441 423 233 222 342 CCC GTA CAT ATC CCC GTA GAA-3' (SEQ ID NO: 19)

Helping primer FSERV: 5-AAA ATT GAA CCG ATC TTA-3' (SEQ ID NO: 20)

Table 5.

FSERVII: 5'-TT CCA TGC TGC ATC GAC ACA GGG AGG CGG CTA TGA TAT
 GAG GAA ATT GCT GAA 344 213 442 342 223 311 431 233 422 411 123
 442 213 122 TGT CGA TAA CCA-3' (SEQ ID NO: 21)

Helping primer RSERVII: 5'- TGT CGA TGC AGC ATG GAA - 3' (SEQ ID NO: 22)

Table 6.

25 FSERIX: 5'-GT CCA AAC ATG GTT TAA GCC 432 243 221 343 222 212
 232 313 114 441 123 244 121 333 TCA GGT TTT CTA CGG GGA-3' (SEQ ID NO: 23)

Helping primer RSERIX: 5'-GGC TTA AAC CAT GTT TGG AC-3' (SEQ ID NO: 24)

30 Distribution of nucleotides in each mutated nucleotide position
 1:97%A, 1%T, 1%C, 1%G
 2:97%T, 1%A, 1%C, 1%G
 3:97%C, 1%A, 1%T, 1%G
 35 4:97%G, 1%A, 1%T, 1%C

Construction of plasmid libraries

Two approximately 1.4 kb fragments were PCR amplified using the primer 1B: 5'-CGA TTG CTG ACG CTG TTA TTT GCG-3' and the random oligonucleotide apparent from table 1, respectively the random oligonucleotide apparent from table 2. The vector 5 pSnK101 and the PCR fragments were digested with EcoRV and EagI for 2 hours. The approximately 3.6 kb vector fragment and the approximately 1.3 kb PCR fragments was purified and ligated overnight and transformed in to *E.coli* and then further transformed into a *Bacillus* host starin as described below. The 10 random oligonucleotides apparent from Tables 3-6 (which by a common term is designated aSER and bSER in Fig. 2) for each region and specific *B. licheniformis* primers 1B (SEQ ID NO: 26) and #63: 5'-CTA TCT TTG AAC ATA AAT TGA AAC C-3' (SEQ ID NO: 27) covering the EcoRV and the EagI sites in the LE174 sequence 15 are used to generate PCR-library-fragments by the overlap extension method (Horton et al., Gene, 77 (1989), pp. 61-68). Figure 2 shows the PCR strategy. The PCR fragments are cloned in the *E. coli/Bacillus* shuttle vector pSNK101 (see Materials and Methods) enabling mutagenesis in *E. coli* and immediate expression in *Bacillus subtilis* preventing lethal accumulation 20 of amylases in *E. coli*. After establishing the cloned PCR fragments in *E. coli*, a modified pUC19 fragment is digested out of the plasmid and the promoter and the mutated Termamyl gene 25 is physically connected and expression can take place in the *Bacillus* host .

Screening

The six libraries were screened in the low pH filter assays described in the "Material and Methods" section above.

30

All variants listed in the table in Example 2 below was prepared as described in Example 1.

EXAMPLE 2

35 Measurement of stability

Normally, industrial liquefaction processes is run at pH 6.0-6.2 with addition of about 40 ppm free calcium in order to

improve the stability at 95°C-105°C. Variants of the invention have been made in order to improve the stability at

1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40ppm free calcium.

5 An assay which measures the stability at acidic pH, pH 5.0, in the presence of 5ppm free calcium was used to measure the increase in stability.

10 10 µg of the variant was incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.0, containing 5ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Results:

15 Increased stability at pH 5.0, 5 ppm calcium incubated at 95°C

MINUTES OF INCUBATION	LE174 WITH K176R+ I201F+ H205N	LE174 WITH K176R+ I201F+ H205N+ E376K+ A420R	LE174 WITH K176R+ I201F+ H205N+ S417T+ A420Q	LE174 WITH K176R+ I201F+ H205N+ S356A+ Y358F
0	100	100	100	100
5	65	61	66	66
10	58	53	60	59
15	51	48	55	56
30	36	39	45	49

Specific activity determination.

20 The specific activity was determined using the Phadebas assay (Pharmacia) (described above) as activity/mg enzyme. The activity was determined using the α-amylase assay described in the Materials and Methods section herein.

LE174 with the following substitutions:

39

K176R+I201F+H205N

Specific activity determined: 13400NU/mg

LE174 with the following substitutions:

5 K176R+I201F+H205N+E376K+A420R:

Specific activity determined: 14770NU/mg

LE174 with the following substitutions:

K176R+I201F+H205N+S417T+A420Q:

10 Specific activity determined: 16670NU/mg

LE174 with the following substitutions:

K176R+I201F+H205N+S356A+Y358F:

Specific activity determined: 15300NU/mg

REFERENCES CITED

- Klein, C., et al., *Biochemistry* 1992, **31**, 8740-8746,
Mizuno, H., et al., *J. Mol. Biol.* (1993) **234**, 1282-1283,
Chang, C., et al., *J. Mol. Biol.* (1993) **229**, 235-238,
5 Larson, S.B., *J. Mol. Biol.* (1994) **235**, 1560-1584,
Lawson, C.L., *J. Mol. Biol.* (1994) **236**, 590-600,
Qian, M., et al., *J. Mol. Biol.* (1993) **231**, 785-799,
Brady, R.L., et al., *Acta Crystallogr. sect. B*, **47**, 527-535,
Swift, H.J., et al., *Acta Crystallogr. sect. B*, **47**, 535-544
- 10 A. Kadziola, Ph.D. Thesis: "An alpha-amylase from Barley and its Complex with a Substrate Analogue Inhibitor Studied by X-ray Crystallography", Department of Chemistry University of Copenhagen 1993
- MacGregor, E.A., *Food Hydrocolloids*, 1987, Vol.1, No. 5-6, p.
- 15 B. Diderichsen and L. Christiansen, Cloning of a maltogenic α -amylase from *Bacillus stearothermophilus*, *FEMS Microbiol. letters*: **56**: pp. 53-60 (1988)
- Hudson et al., *Practical Immunology*, Third edition (1989), Blackwell Scientific Publications,
- 20 Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989
- S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters **22**, 1981, pp. 1859-1869
- Matthes et al., The EMBO J. **3**, 1984, pp. 801-805.
- 25 R.K. Saiki et al., Science **239**, 1988, pp. 487-491.
Morinaga et al., (1984, *Biotechnology* **2**:646-639)
Nelson and Long, Analytical Biochemistry **180**, 1989, pp. 147-151
- Hunkapiller et al., 1984, *Nature* **310**:105-111
- R. Higuchi, B. Krummel, and R.K. Saiki (1988). A general method
30 of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucl. Acids Res.* **16**:7351-7367.
- Dubnau et al., 1971, J. Mol. Biol. **56**, pp. 209-221.
- Gryczan et al., 1978, J. Bacteriol. **134**, pp. 318-329.
- 35 S.D. Erlich, 1977, Proc. Natl. Acad. Sci. **74**, pp. 1680-1682.
- Boel et al., 1990, Biochemistry **29**, pp. 6244-6249.

CLAIMS

1. A variant of a parent Termamyl-like α -amylase, which variant α -amylase has been altered in comparison to the parent α -amylase in one or more solvent exposed amino acid residues on the surface of the α -amylase to increase the overall hydrophobicity of the α -amylase and/or to increase the overall numbers of methyl groups in the sidechains of said solvent exposed amino acid residues on the surface.

10

2. The variant according to claim 1, wherein one or more solvent exposed amino acid residues on a concav surface with inwards bend are altered to more hydrophobic amino acid residues.

3. The variant according to claim 1, wherein one or more solvent exposed amino acid residues on a convex surface are altered to increase the number of methyl groups in the sidechain.

4. A variant of a parent Termamyl-like α -amylase, comprising an alteration at one or more positions selected from the group of:

E376, S417, A420, S356, Y358;
wherein (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position.

25 (ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid.

(b) the variant has α -amylase activity and (c) each position
30 corresponds to a position of the amino acid sequence of the
parent Termamyl-like α -amylase having the amino acid sequence of
SEQ ID NO: 4.

5. The variant according to claim 4, which variant has an
35 alteration in one or more solvent exposed amino acid residues as
defined in any of claims 1-3.

6. The variant of any of claims 1-5, wherein the parent Termamyl-like α -amylase is derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. stearothermophilus*, *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375.
7. The variant according to claim 6, wherein the parent α -amylase is derived from *B. licheniformis* strain ATCC 27811.
8. The variant according to claims 1-6, wherein the parent Termamyl-like α -amylase is any of the α -amylases selected from the group depicted in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, and 8.
9. The variant according to any of claims 1-8, wherein the parent Termamyl-like α -amylase has an amino acid sequence which has a degree of identity to SEQ ID NO: 4 of at least 65%, preferably 70%, more preferably at least 80%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 97%, and even more preferably at least 97%.
10. The variant according to any of claims 1-10, wherein the parent Termamyl-like α -amylase is encoded by a nucleic acid sequence which hybridizes under medium, preferred high stringency conditions, with the nucleic acid sequence of SEQ ID NO: 12.
11. The variant according to claims 1-10, wherein the parent Termamyl-like α -amylase is a hybrid of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5.
12. The variant according to claim 11, wherein the parent hybrid Termamyl-like α -amylase is LE174.

13. The variant according to any of claims 1-12, wherein the parent α -amylase further has a mutation in one or more of the following positions: K176, I201 and H205 (using the numbering in SEQ ID NO: 4).
- 5
14. The variant according to claim 13, wherein the parent α -amylase has one or more the following substitutions: K176R, I201F and/or H205N (using the numbering in SEQ ID NO: 4).
- 10 15. The variant according to claim 14, wherein the parent α -amylase has the following substitutions: K176R+I201F+H205N (using the numbering in SEQ ID NO: 4).
- 15 16. The variant according to claims 1 to 15, wherein the variant has increased stability at pHs below 7.0 (acidic pH) and/or at low calcium concentration and/or at temperatures in the range from 95 to 160°C (high temperatures) relative to the parent α -amylase.
- 20 17. The variant according to any of claims 1 to 16, which variant has one or more of the following substitutions: E376K, S417T, A420Q, R, S356A, Y358F.
- 25 18. A DNA construct comprising a DNA sequence encoding an α -amylase variant according to any one of claims 1 to 17.
19. A recombinant expression vector which carries a DNA construct according to claim 18.
- 30 20. A cell which is transformed with a DNA construct according to claim 18 or a vector according to claim 19.
21. A cell according to claim 20, which is a microorganism.
- 35 22. A cell according to claim 21, which is a bacterium or a fungus.

23. The cell according to claim 22, which is a grampositive bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*,
s *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus* or *Bacillus thuringiensis*.
24. A detergent additive comprising an α -amylase variant according to any one of claims 1 to 17, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.
25. A detergent additive according to claim 24 which contains 0.02-200 mg of enzyme protein/g of the additive.
26. A detergent additive according to claims 24 or 25, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.
27. A detergent composition comprising an α -amylase variant according to any of claims 1 to 17.
28. A detergent composition according to claim 27 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.
29. A manual or automatic dishwashing detergent composition comprising an α -amylase variant according to any of claims 1 to 17.
30. A dishwashing detergent composition according to claim 29 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.
31. A manual or automatic laundry washing composition comprising

an α -amylase variant according to any of claims 1 to 17.

32. A laundry washing composition according to claim 31, which additionally comprises another enzyme such as a protease, a s lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

33. A composition comprising:

(i) a mixture of the α -amylase from *B. licheniformis* having the sequence shown in SEQ ID NO: 4 with one or more variants 10 according to any of claims 1 to 17 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3; or
15 (ii) a mixture of the α -amylase from *B. stearothermophilus* having the sequence shown in SEQ ID NO: 3 with one or more variants according to any of claims 1 to 17 derived from one or more other parent Termamyl-like α -amylases; or
20 (iii) a mixture of one or more variants according any of claims 1 to 17 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 with one or more variants according to the invention derived from one or more other parent Termamyl-like α -amylases.

34. The composition comprising a variant of any of claims 1 to 17 wherein the parent α -amylase is a hybrid alpha-amylase 25 comprising a N-terminal part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a C-terminal part of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4.

35. The composition according to claim 34, wherein the parent 30 hybrid Termamyl-like α -amylase is LE174

36. The composition according to claims 35, wherein the parent Termamyl-like α -amylase is LE174 with an alteration in one or more of the following positions: K176, I201 and H205.

37. The composition according to claims 36, wherein the parent Termamyl-like α -amylase is LE174 with one or more of the following substitutions: K176R, I201F and H205N.

5

38. Use of an α -amylase variant according to any one of claims 1 to 17 or a composition according to claims 33 to 37 for washing and/or dishwashing.

10 39. Use of an α -amylase variant according to any one of claims 1 to 17 or a composition according to claims 33 to 37 for textile desizing.

D
E
S
G
S
D

40. Use of an α -amylase variant according to any of claims 1 to 17 or a composition according to claims 33 to 37 for starch liquefaction.

41. A method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased stability at high temperatures relative to the parent, the method comprising:

- (a) subjecting a DNA sequence encoding the parent Termamyl-like α -amylase to random mutagenesis,
15 (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
20 (c) screening for host cells expressing a mutated α -amylase which has increased stability at high temperatures relative to the parent Termamyl-like α -amylase.

Title: α -amylase variants

ABSTRACT

The invention relates to a variant of a parent Termamyl-like

α -amylase, comprising mutations in two, three, four, five or six regions/positions. The variants have increased stability at high temperatures (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an α -amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an α -amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an α -amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an α -amylase variant of the invention, a method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased.

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50

1 HHNGTNGTMM QYFEWHLPND GNHWNRLRDD ASNLRNRGIT AIWIPPAWKG
 2 . NGTNGTMM QYFEWYL PND GNHWNRLRSD ASNLKDKGIS AVWIPPAWKG
 3 HHNGTNGTMM QYFEWYL PND GNHWNRLRDD AANLKSKGIT AVWIPPAWKG
 4 . . VNGTLM QYFEWYTPND GQHWKRLQND AEHLSDIGIT AVWIPPAYKG
 5 . . ANLNGLTM QYFEWYMPND GQHWRRRLQND SAYLAEHGIT AVWIPPAYKG
 6 . AAPFNGTMM QYFEWYL PDD GTLWTKVANE ANNLSLIGIT ALWLPPAYKG

10

51

100

1 TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TRSQLESAIH ALKNNGVQVY
 2 ASQNDVGYGA YDLYDLGEFN QKGTVRTKYG TRNQLQAAVN ALKSNGIQVY
 3 TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TRNQLQAAVT SLKNNGIQVY
 15 4 LSQSDNGYGP YDLYDLGEFQ QKGTVRTKYG TKSELQDAIG SLHSRNVQVY
 5 TSQADVGYGA YDLYDLGEFH QKGTVRTKYG TKGELQSAIK SLHSRDINVY
 6 TSRSDVGYGV YDLYDLGEFN QKGTVRTKYG TKAQYLOAIQ AAHAAGMQVY

101

150

20 1 GDVVMNHKGG ADATENVLAV EVNPNNRNQE ISGDYTI EAW TKFD PGRGN
 2 GDVVMNHKGG ADATEMVR AVEVNPNNRNQE VSGEYTI EAW TKFD PGRGN
 3 GDVVMNHKGG ADGTEIVNAV EVNRSNRNQE TSGEYAI EAW TKFD PGRGN
 4 GDVVLNHKAG ADATEDVTAV EVNPANRNQE TSEYQIKAW TDFRFPGRGN
 5 GDVVINHKGG ADATEDVTAV EVDPADRNRV ISGEHLIKAW THFHF PGRGS
 25 6 ADVVF DHKGG ADGTEWVD AVEVNPSDRNQE ISGTYQI QAW TKFD PGRGN

151

200

1 TYSDFKWRWY HFDGVWDQ S RQFQNRIYKF RGDGKA WDW VDSENGNYDY
 2 THSNFKWRWY HFDGVWDQ S RKLNNRIYKF RGDGKGWDWE VDTENGNYDY
 30 3 NHSSFKWRWY HFDGTWDQ S RQLQNKIYKF RGTGKA WDW VDTENGNYDY
 4 TYSDFKWHWY HFDGADWDE S RKL.SRIPKF RGEGKA WDW VSSENGNYDY
 5 TYSDFKWHWY HFDGTWDDE S RKL.NRIYKF .QGKA WDW VSNENGNYDY
 6 TYSSEFKWRWY HFDGVWDDE S RKL.SRIYKF RGIGKA WDW VDTENGNYDY

Fig. 1

2/4

5	201	250
1	LMYADVDMDH PEVVNELRRW GEWYTNTLNL DGFRIDAVKH IKYSFTRDWL	
2	LMYADIDMDH PEVVNELRNW GWYWTNTLGL DGFRIDAVKH IKYSFTRDWS	
3	LMYADVDMDH PEVIHELRNW GWYWTNTLNL DGFRIDAVKH IKYSFTRDWL	
4	LMYADVDYDH PDVVAETKKW GIWYANELSL DGFRIDAACKH IKFSFLRDWV	
10 5	LMYADIDYDH PDVAAEIKRW GTWYANELQL DGFRLDRAVKH IKFSFLRDWV	
6	LMYADLDMHD PEVVTELKNW GKWYVNTTNI DGFRLDRAVKH IKFSFFPDWL	
20	251	300
1	THVRNATGKE MFAVAEFWKN DLGALENYLN KTNWNHSVFD VPLHYNLYNA	
15 2	IHVRSATGKN MFAVAEFWKN DLGAIENYLN KTNWNHSVFD VPLHYNFYNA	
3	THVRNTTGKP MFAVAEFWKN DLGAIENYLN KTSWNHSAFD VPLHYNLYNA	
4	QAVRQATGKE MFTVAEYWQN NAGKLENYLN KTSFNQSVFD VPLHFNLQAA	
5	NHvrektgke MFTVAEYWQN DLGALENYLN KTNFNHSVFD VPLHYQFHAA	
6	SYVRSQTGKP LFTVGEYWSY DINKLHNYIT KTDGTMISLD APLHNKFYTA	
20	301	350
1	SNSGGNYDMA KLLNGTVVQK HPMHAVTFVD NHDSQPGESL ESFVQEWFKP	
2	SKSGGNYDMR QIFNGTVVQR HPMHAVTFVD NHDSQPEEAL ESFVEEWFKP	
3	SNSGGYYDMR NILNGSVVQK HPTHAVTFVD NHDSQPGEAL ESFVQQWFKP	
25 4	SSQGGGYDMR RLLDGTVVSR HPEKAVTFVE NHDTQPGQSL ESTVQTWFKP	
5	STQGGGYDMR KLLNGTVVSK HPLKSVTFVD NHDTQPGQSL ESTVQTWFKP	
6	SKSGGAFDMR TLMTNTLMKD QPTLAVTFVD NHDTEPGQAL QSWVDPWFKP	
30	351	400
1	LAYALILTRE QGYPYVFYGD YYGIPTHS.. VPAMKAKID PILEARQNFA	
2	LAYALTLTRE QGYPYVFYGD YYGIPTHG.. VPAMKSKID PILEARQKYA	
3	LAYALVLTRE QGYPYVFYGD YYGIPTHG.. VPAMKSKID PLLQARQTFA	
4	LAYAFILTRE SGYPQVFYGD MYGTKGTSPK EIPSLKDNE PILKARKEYA	
5	LAYAFILTRE SGYPQVFYGD MYGTKGDSQR EIPALKHKIE PILKARKQYA	
35 6	LAYAFILTRQ EGYPYVFYGD YYGIPQYN.. IPSLKSID PLIARRDYA	
40	401	450
1	YGTQHDYFDH HNIIGWTREG NTTHPNNSGLA TIMSDGPGGE KWMYVGQNK	
2	YGRQN	
40 3	YGTQHDYFDH HDIIGWTREG NSSHPNSGLA TIMSDGPGGN KWMYVGKNKA	
4	YGPQHDYIDH PDVIGWTREG DSSAAKSGLA ALITDGPSS KRMYAGLKNA	
5	YGAQHDYFDH HDIVGWTREG DSSVANSGLA ALITDGPSS KRMYVGRQNA	
6	YGTQHDYLDH SDIIGWTREG GTEKPGSGLA ALITDGPSS KWMYVGKQHA	

Fig. 1 (continued)

5709.204-WO

3/4

	451	500
5 1	GQVWHDITGN KPGTVTINAD GWANFSVNNGG SVSIWVKR.	.
2	.	.
3	GQVWRDITGN RTGTVTINAD GWGNFSVNNGG SVSVWVKQ.	.
4	GETWYDITGN RSDTVKIGSD GWGEFHVNNDG SVSIYVQ.	.
5	GETWHDITGN RSEPVVINSE GWGEFHVNNGG SVSIYVQR.	.
10 6	GKVFYDLTGN RSDTVTINSD GWGEFKVNNGG SVSVWVPRKT TVSTIARPIT	
	501	519
1	.	.
2	.	.
15 3	.	.
4	.	.
5	.	.
6	TRPWTGEFVR WTEPRLVAW	

Fig. 1 (continued)

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5709.204-WO

NOVO NORDISK PAT DP +45 44493256

NO. 2740 P. 26/50

4/4

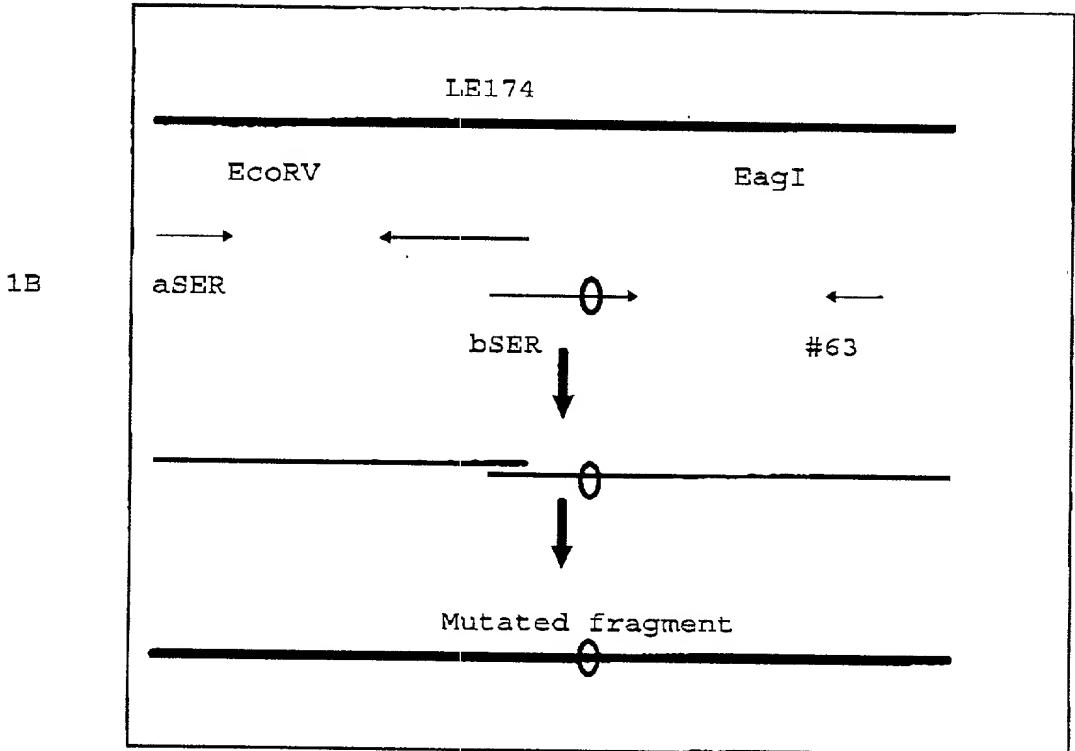


Fig. 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
10 (G) TELEPHONE: +45 44 44 88 88
(H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: α -amylase variants

(iii) NUMBER OF SEQUENCES: 32

(iv) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(2) INFORMATION FOR SEQ ID NO: 1:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (iii) MOLECULE TYPE: protein

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
20 25 30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
115 120 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
165 170 175

Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

65 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220

5 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
 245 250 255

10 Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
 275 280 285

15 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300

20 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
 305 310 315 320

His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335

25 Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
 340 345 350

Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365

30 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400

Gln His Asp Tyr Phe Asp His Asp Ile Ile Gly Trp Thr Arg Glu
 405 410 415

35 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
 435 440 445

40 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460

45 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480

Val Trp Val Lys Gln
 485

- 55 (2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 60 (ii) MOLECULE TYPE: protein
 (iii) Organism: *Bacillus* sp.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

65 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15

3

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
20 25 30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
5 35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
10 65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
85 90 95

15 Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
20 115 120 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
25 130 135 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
30 165 170 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
35 180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
40 195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
45 210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
50 225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
55 245 250 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
60 260 265 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
65 275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
70 290 295 300

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
75 305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
80 325 330 335

Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
85 340 345 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
90 355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala

370

375

380

5 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
 385 390 395 400

Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415

10 Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
 435 440 445

15 Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
 450 455 460

20 Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480

Ile Trp Val Lys Arg
 485

25 (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 514 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein
 (iii) Organism: *Bacillus stearothermophilus*.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

35 Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
 1 5 10 15

40 Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
 20 25 30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
 35 40 45

45 Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
 50 55 60

Leu Gly Glu Phe Asn Gln Lys Gly Ala Val Arg Thr Lys Tyr Gly Thr
 65 70 75 80

50 Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
 85 90 95

55 Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
 100 105 110

60 Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
 115 120 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
 130 135 140

65 Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
 145 150 155 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
 165 170 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu

5

180	185	190
-----	-----	-----

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His 195	200	205
--	-----	-----

5 Pro Glu Val Val Thr Glu Leu Lys Ser Trp Gly Lys Trp Tyr Val Asn 210	215	220
--	-----	-----

10 Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys 225	230	235
---	-----	-----

Phe Ser Phe Phe Pro Asp Trp Leu Ser Asp Val Arg Ser Gln Thr Gly 245	250	255
--	-----	-----

15 Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys 260	265	270
---	-----	-----

Leu His Asn Tyr Ile Met Lys Thr Asn Gly Thr Met Ser Leu Phe Asp 275	280	285
--	-----	-----

20 Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Thr 290	295	300
---	-----	-----

25 Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro 305	310	315
---	-----	-----

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln 325	330	335
--	-----	-----

30 Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala 340	345	350
---	-----	-----

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp 355	360	365
--	-----	-----

35 Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile 370	375	380
---	-----	-----

40 Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His 385	390	395
---	-----	-----

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val 405	410	415
--	-----	-----

45 Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro 420	425	430
---	-----	-----

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val 435	440	445
--	-----	-----

50 Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser 450	455	460
---	-----	-----

55 Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp 465	470	475
---	-----	-----

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Trp Ser Ile Thr Thr 485	490	495
--	-----	-----

60 Arg Pro Trp Thr Asp Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val 500	505	510
---	-----	-----

Ala Trp

65 (2) INFORMATION FOR SEQ ID NO: 4:
 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 483 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein
(iii) Organism: *Bacillus licheniformis*
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
10 1 5 10 15

Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
20 25 30

15 Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
35 40 45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
50 55 60

20 Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65 70 75 80

25 Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
85 90 95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
100 105 110

30 Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
115 120 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
35 130 135 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145 150 155 160

40 Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
165 170 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
45 180 185 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
195 200 205

50 Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
210 215 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
55 225 230 235 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
245 250 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
60 260 265 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
275 280 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
65 290 295 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305 310 315 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
325 330 335

5 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
340 345 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
10 355 360 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
370 375 380

15 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385 390 395 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
20 405 410 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
420 425 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
25 435 440 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
450 455 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
30 465 470 475 480

Val Gln Arg

35 (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 480 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) Organism: *Bacillus amylcliquefaciens*
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

45 Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
1 5 10 15

Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
50 20 25 30

Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser
55 35 40 45

Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu
60 50 55 60

Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
65 65 70 75 80

Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr
70 85 90 95

Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
75 100 105 110

85 Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser
90 115 120 125

Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg
 130 135 140

5 Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly
 145 150 155 160

Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg
 165 170 175

10 Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn
 180 185 190

15 Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val
 195 200 205

Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser
 210 215 220

20 Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe
 225 230 235 240

25 Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met
 245 250 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn
 260 265 270

30 Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
 275 280 285

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Tyr Asp Met
 290 295 300

35 Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
 305 310 315 320

40 Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335

45 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365

50 Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
 370 375 380

55 Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
 385 390 395 400

Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
 405 410 415

60 Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430

Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
 435 440 445

65 Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
 450 455 460

Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
 465 470 475 480

10

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
 290 295 300

5 Gly Asn Tyr Asp Met Arg Asn Ile Phe Asn Gly Thr Val Val Gln Arg
 305 310 315 320

His Pro Ser His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335

10 Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
 340 345 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365

15 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
 370 375 380

20 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys
 385 390 395 400

Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415

25 Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430

30 Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
 435 440 445

Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460

35 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480

Ile Trp Val Asn Lys
 485

40 (2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 485 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

50 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
 20 25 30

55 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60

60 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80

65 Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 85 90 95

11

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
 5 115 120 125
 Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140
 10 Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
 165 170 175
 15 Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190
 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 20 195 200 205
 Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220
 25 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
 30 245 250 255
 Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
 35 275 280 285
 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 40 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
 305 310 315 320
 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 45 325 330 335
 Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
 340 345 350
 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 50 355 360 365
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380
 55 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400
 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
 60 405 410 415
 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
 65 435 440 445
 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile

450

455

460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 5 Val Trp Val Lys Gln
 485

(2) INFORMATION FOR SEQ ID NO: 8:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein
 (iii) Organism: *Bacillus* sp.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

20 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15

25 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30

30 Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
 35 40 45

35 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60

40 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80

45 Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95

50 Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110

55 Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
 115 120 125

60 Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140

65 Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
 145 150 155 160

70 His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
 165 170 175

75 Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190

80 Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 195 200 205

85 Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
 210 215 220

90 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240

95 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
 245 250 255

100 Thr Gly Lys Glu Met Phe Ala Val Ala Gln Phe Trp Lys Asn Asp Leu

13

	260	265	270
	Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val		
5	275	280	285
	Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly		
	290	295	300
10	Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys		
	305	310	315
	His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro		
	325	330	335
15	Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala		
	340	345	350
	Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr		
20	355	360	365
	Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala		
	370	375	380
25	Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr		
	385	390	395
	Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu		
	405	410	415
30	Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp		
	420	425	430
	Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly		
35	435	440	445
	Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile		
	450	455	460
40	Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser		
	465	470	475
	Ile Trp Val Lys Arg		
	485		

45 (2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

55	CATCATAATG GAACAAATGG TACTATGATG CAATATTCG AATGGTATTT GCCAAATGAC	60
	GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120
60	GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC	180
	TATGATTTAT ATGATCTTGG AGAGTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA	240
	ACACGCAACC AGCTACAGGC TCGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGGTATAT	300
65	GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCCGTA	360
	GAAGTGAAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG	420

	ACAAAAGTTG ATTTCTGG AAGAGGAAAT AACCAATTCCA GCTTTAAGTG GCGCTGGTAT	480
5	CATTTTGATG GGACAGATTG GGATCAGTC CGCCAGCTC AAAACAAAAT ATATAAATTC	540
	AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT	600
	CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG	660
10	GGAGTGTGGT ATACGAATAAC ACTGAACCTT GATGGATTAA GAATAGATGC AGTGAACAT	720
	ATAAAATATA GCTTTACGAG AGAATGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA	780
15	ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTCTG CAATTGAAAA CTATTGAAAT	840
	AAAACAAGTT GGAATCACTC GGTSTTTGAT GTTCCTCTCC ACTATAATT GTACAATGCA	900
	TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTAA ATGGTTCTGT GGTGCAAAAA	960
20	CATCCAACAC ATGCCGTTAC TTTTGTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG	1020
	GAATCCTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA	1080
25	CAAGGTTATC CTTCCGTATT TTATGGGAT TACTACGGTA TCCCAACCCA TGGTGTCCG	1140
	GCTATGAAAT CTAAAATAGA CCCTCTCTG CAGGCACGTC AAACCTTTGC CTATGGTACG	1200
	CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC	1260
30	CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG	1320
	TATGTGGGAA AAAATAAACG GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC	1380
	ACCGTCACAA TTAATGCAGA CGGATGGGAA ATTTCCTCTG TTAATGGAGG GTCCGTTCG	1440
35	GTGGGGTGA AGCAA	1455

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 1455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

	CATCATAATG GGACAAATGG GACGATGATG CAATACCTTG AATGGCACTT GCCTAATGAT	60
50	GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC	120
	GCTATTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAATGATGTGGG GTATGGAGCC	180
	TATGATCTT ATGATTTAGG GGAATTAAAT CAAAACGGGA CGGTTCTGAC TAAGTATGGG	240
55	ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTAT	300
	GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC	360
60	GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGG ACTACACAAT TGAGGCTTGG	420
	ACTAAGTTG ATTTCCAGG GAGGGTAAAT ACATACTCAG ACTTAAATG GCGTTGGTAT	480
	CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC	540
65	CGAGGTGATG CTAAGGCATG GGATTGGGAA GTAGATTGGG AAAATGGAAA TTATGATTAT	600

15

TTAATGTATG	CAGATGTAGA	TATGGATCAT	CCGGAGGTAG	TAATGAGCT	TAGAAGATGG	660	
GGAGAACATGGT	ATACAAARTAC	ATTAAATCTT	GATGGATTAA	GGATCGATGC	GGTGAAGCAT	720	
5	ATTAAATATA	GCTTTACACG	TGATTGGTTG	ACCCATGTAA	GAAACGCAAC	GGGAAAAGAA	780
ATGTTTGCTG	TTGCTGAATT	TTGGAAAAAT	GATTTAGGTG	CCTTGGAGAA	CTATTTAAAT	840	
AAAACAAACT	GGAATCATTC	TGTCTTTGAT	GTCCCCCTTC	ATTATAATCT	TTATAACGCG	900	
10	TCAAATAGTG	GAGGCCAACTA	TGACATGGCA	AAACTCTTA	ATGGAACGGT	TGTTCAAAAG	960
CATCCAATGC	ATGCCGTAAC	TTTGTGGAT	AATCACGATT	CTCAACCTGG	GGAATCATTA	1020	
15	GAATCATTG	TACAAGAATG	GTAAAGCCA	CTTGCTTATG	CGCTTATTTT	AAACAAGAGAA	1080
CAAGGCTATC	CCTCTGTCTT	CTATGGTAC	TACTATGGAA	TTCCAACACA	TAGTGTCCC	1140	
GCAATGAAAG	CCAAGATTGA	TCCAATCTTA	GAGGCCGTC	AAAATTTGC	ATATGGAACA	1200	
20	CAACATGATT	ATTTTGACCA	TCATAATATA	ATCGGATGGA	CACGTGAAGG	AAATACCACG	1260
CATCCCAATT	CAGGACTTGC	GACTATCATG	TCGGATGGSC	CAGGGGGAGA	GAAATGGATG	1320	
25	TACGTAGGGC	AAAATAAAAGC	AGGTCAAGTT	TGGCATGACA	TAACTGGAAA	AAACCAGGA	1380
ACAGTTACGA	TCAATGCAGA	TGGATGGGCT	AATTTTCAG	TAATGGAGG	ATCTGTTCC	1440	
30	ATTTGGGTGA	AACGA				1455	

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1548 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: *Bacillus stearothermophilus*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGCACCGT	TTAACGGCAC	CATGATGCAG	TATTTGAAAT	GGTACTTGCC	GGATGATGGC	60	
ACGTTATGGA	CCAAAGTGGC	CAATGAAGCC	AAACAATTAT	CCAGCCTTGG	CATCACCGCT	120	
45	CTTTGGCTGC	CGCCCCCTTA	CAAAGGAACA	AGCCGCAGCG	ACGTAGGGTA	CGGAGTATAAC	180
GACTTGTATG	ACCTCGGCAG	ATTCAATCAA	AAAGGGACCG	TCCGCACAAA	ATACGGAAACA	240	
50	AAAGCTCAAT	ATCTTCAAGC	CATTCAAGCC	GCCCACGCCG	CTGGAATGCA	AGTGTACGCC	300
GATGTCGTGT	TCGACCATAA	AGGCCGGCGCT	GACGGCACCG	AATGGGTGGA	CGCCGTCGAA	360	
55	GTCAATCCGT	CCGACCGCAA	CCAAGAAATC	TCGGGCACCT	ATCAAATCCA	AGCATGGACG	420
AAATTTGATT	TTCCCGGGCG	GGGCAACACC	TACTCCAGCT	TTAAGTGGCG	CTGGTACCAT	480	
60	TTTGACGGCG	TTGATTGGGA	CGAAAGCCGA	AAATTGAGCC	GCATTTACAA	ATTCCGGCGC	540
ATCGGCAAAG	CGTGGGATTG	GGAAAGTAGAC	ACGGAAAACG	GAAACTATGA	CTACTTAATG	600	
TATGCCGACC	TTGATATGGA	TCATCCCGAA	GTCGTGACCG	AGCTGAAAAAA	CTGGGGAAA	660	
65	TGGTATGTCA	ACACAAACGAA	CATTGATGGG	TTCCGGCTTG	ATGCCGTCAA	GCATATTAAG	720
TTCAGTTTTT	TTCCCTGATTG	GTTGTCGTAT	GTGCGTTCTC	AGACTGGCAA	GCCGCTATTT	780	

16

ACCGTCGGGG	AATATTGGAG	CTATGACATC	AACAAGTGC	ACAATTACAT	TACGAAAACA	940	
GACGGAAACGA	TGTCTTGTT	TGATGCCCG	TTACACAAACA	AATTTTATAC	CGCTTCCAAA	900	
5	TCAGGGGGCG	CATTTGATAT	GCGCACGTTA	ATGACCAATA	CTCTCATGAA	AGATCAACCG	960
ACATTGGCCG	TCACCTTCGT	TGATAATCAT	GACACCGAAC	CCGGCCAAGC	GCTGCAGTCA	1020	
TGGGTCGACC	CATGGTCAA	ACCGTTGGCT	TACGCCCTTA	TTCTAACTCG	GCAGGAAGGA	1080	
10	TACCCGTGCG	TCTTTTATGG	TGACTATTAT	GGCATTCCAC	AATATAACAT	TCCTTCGCTG	1140
AAAAGCAAAA	TCGATCCGCT	CCTCATCGCG	CGCAGGGATT	ATGCTTACGG	AACGCAACAT	1200	
15	GATTATCTTG	ATCACTCCGA	CATCATCGGG	TGGACAAACGG	AACGGGGCAC	TGAAAAACCA	1260
GGATCCGGAC	TGGCCGCACT	GATCACCGAT	GGGCCGGGAG	GAAGCAAATG	GATGTACGTT	1320	
20	GGC _n AAACAAC	ACGCTGGAAA	ACTGTTCTAT	GACCTTACCG	GCAACCGGAG	TGACACCGTC	1380
ACCATCAACA	GTGATGGATG	GGGGGAAITTC	AAAGTCAATG	GGGGTTCGGT	TTCGGTTTGG	1440	
25	GTTCCTAGAA	AAACGACCGT	TTCTACCATC	GCTCGGCCGA	TCACAAACCCG	ACCGTGGACT	1500
GGTGAATTCTG	TCCGTTGGAC	CGAACACACGG	TTGGTGGCAT	GGCCTTGA		1548	

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 1920 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) Organism: *Bacillus licheniformis*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 421..1872

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

40	CGGAAGATTG	GAAGTACAAA	AATAAGCAAA	AGATTGTC	TCATGTCATG	AGCCATGCGG	60
	GAGACGGAAA	AATCGTCTTA	ATGCACGATA	TTTATGCAAC	GTTCGCGAGAT	GCTGCTGAAG	120
45	AGATTATTAA	AAAGCTGAAA	GCAAAAGGCT	ATCAATTGCT	AACTGTATCT	CAGCTTGAAG	180
	AAGTGAAGAA	GCAGAGAGGC	TATTGAATAA	ATGAGTAGAA	GCCCATATC	GGCGCTTTTC	240
50	TTTGGAAAGA	AAATATAGGG	AAAATGGTAC	TTGTTAAAAA	TTCGGAATAT	TTATACAACA	300
	TCATATGTTT	CACATTGAAA	GGGGAGGAGA	ATCATGAAAC	AACAAAACG	GCTTTACGCC	360
	CGATTGCTGA	CGCTGTTATT	TGCGCTCATC	TTCTGCTGC	CTCATTCTGC	AGCAGCGGCC	420
55	GCA AAT CTT	AAT GGG ACG	CTG ATG CAG	TAT TTT GAA	TGG TAC ATG	CCC	468
	AAT GAC GGC	CAA CAT TGG	AGG CGT TTG	CAA AAC GAC	TCG GCA TAT	TTG	516
	GCT GAA CAC	GGT ATT ACT	GCC GTC TGG	ATT CCC CCG	GCA TAT AAG	GGA	564
60	ACG AGC CAA	GCG GAT GTG	GGC TAC GGT	GCT TAC GAC	CTT TAT GAT	TTA	612
	GGG GAG TTT	CAT CAA AAA	GGG ACG GTT	CGG ACA AAG	TAC GGC ACA	AAA	660
65	GGA GAG CTG	CAA TCT GCG	ATC AAA AGT	CTT CAT TCC	CGC GAC ATT	AAC	708
	GTT TAC GGG	GAT GTG GTC	ATC AAC CAC	AAA GCC GCT	GAT GCG ACC		756

GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA	804
5 ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG	852
GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT	900
GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG	948
10 TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC	996
TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC	1044
GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA	1092
15 15 TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT	1140
TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG	1188
20 TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC	1236
TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT	1284
CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG	1332
25 AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG	1380
GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG	1428
30 TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC	1476
ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG	1524
ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT	1572
35 GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT	1620
GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC	1668
40 AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC	1716
GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GGC GGT GAG ACA	1764
TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC ATT TCG	1812
45 GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT	1860
GTT CAA AGA TAG AAGAGCAGAG AGGACGGATT TCCTGAAGGA AATCCGTTTT	1912
50 TTTATTTT	1920

(2) INFORMATION FOR SEQ ID NO: 13:

55 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATCATAATG GAACAAATGG TACTATGATG CAATATTCG AATGGTATTT GCCAAATGAC	60
65 GGGAAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120

GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTA GG TTATGGAGCC 180
 TATGATTTAT ATGATCTTGG AGAGTTAAC CAGAAGGGG CGGTTCGTAC AAAATATGGA 240
 5 ACACGCAACC AGCTACAGGC TGCAGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300
 GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360
 10 GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420
 15 ACAAAAGTTTG ATTTTCTTGG AAGAGGAAAT AACCAATTCCA GCTTTAAGTG GCGCTGGTAT 480
 CATTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTG 540
 20 AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600
 CTTATGATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660
 25 GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTAA GAATAGATGC AGTGAACAT 720
 ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCCAC AGGTAAACCA 780
 ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTGGTG CAATTGAAAA CTATTGAAAT 840
 30 AAAACAAGTT GGAATCACTC GGTGTTGAT GTTCCTCTCC ACTATAATT GTACAATGCA 900
 TCTAATAGCG GTGGTTAATTA TGATATGAGA AATATTAAAT ATGGTTCTGT GGTGAAAAAA 960
 CATCCAACAC ATGCCGTTAC TTTTGTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020
 35 GAATCCTTGT TTCAACAATG GTTIAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080
 CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTCCG 1140
 40 GCTATGAAAT CTAAAATAGA CCCTCTCTG CAGGCACGTC AAACCTTGC CTATGGTACG 1200
 CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260
 CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320
 45 TATGTGGGGAA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380
 ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTCTCTG TTAATGGAGG GTCCGTTCG 1440
 GTTTGGGTGA AGCAA 1455

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTGG AATGGCACTT GCCTAATGAT 60
 50 GGGAAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120
 GCTATTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAATGATGTGGG GTATGGAGCC 180
 TATGATCTT ATGATTTAGG GGAATTTAAT CAAAAGGGG CGGTTCGTAC TAAGTATGGG 240
 65 ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTAT 300
 GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360

GAGGTGAATC CAAATAACCG	GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG	420
ACTAAGTTTG ATTTTCAGG GAGGGGTAAT ACATACTCAG	ACTTAAATG GCGTTGGTAT	480
5 CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT	CTACAAATTC	540
CGAGGTGATG GTAAGGCATG GGATTGGAA GTAGATTCCG AAAATGGAAA TTATGATTAT		600
10 TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG		660
GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT		720
15 ATTAATATA GCTTACACG TGATTGGTTG ACCCATGTAAC GAAACGCAAC GGGAAAAGAA		780
ATGTTGCTG TTGCTGAATT TTGAAAAAT GATTAGGTG CCTTGGAGAA CTATTTAAAT		840
AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG		900
20 TCAAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAAG		960
CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA		1020
GAATCATTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT ACAAGAGAA		1080
25 CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGAA TTCCAACACA TAGTGTCCCA		1140
GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTGC ATATGGAACA		1200
30 CAACATGATT ATTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG		1260
CATCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG		1320
TACGTAGGGC AAAATAAACG AGGTCAAGTT TGGCATGACA TAATGGAAA TAAACCAGGA		1380
35 ACAGTTACGA TCAATGCAGA TGGATGGCT AATTTTCAG TAAATGGAGG ATCTGTTCC		1440
ATTGGGTGA AACGA		1455

40 (2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 74 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (ix) FEATURE:
 - (A) NAME/ KEY: misc-feature:
 - (B) OTHER INFORMATION: /desc = "RSERI"
 - (ix) FEATURE:
 - (A) NAME/KEY: misc-feature
 - (B) LOCATION: 21-62
 - (D) OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
GCGTTTGCC GGCGACATA 3122343222 4333313344

60 4233423242 2122112433 43CAAACCTG AATT 74

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 122 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

20

(D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "RSERII"
 (ix) FEATURE:
 (A) NAME/KEY: misc-feature
 (B) LOCATION: 63-104
 (D) OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
 10 2:97%T, 1%A, 1%C, 1%G
 15 3:97%C, 1%A, 1%T, 1%G
 4:97%G, 1%A, 1%T, 1%C
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:
 GCGTTTGCC GGCGACATA CATTGCTTT GCCCCACCGG GTCCGTCTGT
 15 TATTAATGCC GC31113324 1122243113 3414324234 3322333224
 2331GCCGAC AATGTCATGG TG
 (2) INFORMATION FOR SEQ ID NO: 17:
 (i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 78 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 25 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "RSERIII"
 (ix) FEATURE:
 (A) NAME/KEY: misc-feature
 30 (B) LOCATION: 19-60
 (D) OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
 2:97%T, 1%A, 1%C, 1%G
 3:97%C, 1%A, 1%T, 1%G
 4:97%G, 1%A, 1%T, 1%C
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:
 GTCGCCCTTC CTTGTCCA43 3413112423 1244244234 1112112312
 4324243233 GTACGCATAC TGTGTTCT
 (2) INFORMATION FOR SEQ ID NO: 18:
 (i) SEQUENCE CHARACTERISTICS:
 40 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 45 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "FSERIII"
 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:
 TGGACAAGGG AAGGCGACAG
 (2) INFORMATION FOR SEQ ID NO: 19:
 (i) SEQUENCE CHARACTERISTICS:
 55 (A) LENGTH: 81 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 60 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "RSERV"
 (ix) FEATURE:
 (A) NAME/KEY: misc-feature
 65 (B) LOCATION: 19-60
 (D) OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
 2:97%T, 1%A, 1%C, 1%G

3:97% C, 1%A, 1%T, 1%G
4:97% G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
TAAGATCGGT TCAATTTT42 4222311443 1441122234 3432444142
5 3233222342 CCCGTACATA TCCCCGTAGA A

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 15 (ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "FSERV"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
AAAATTGAAC CGATCTTA

18

20

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
25 (A) LENGTH: 107 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "FSERVII"

(ix) FEATURE:

- (A) NAME/KEY: misc-feature

(B) LOCATION: 54-95

- 35 (D) OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

40 TTCCATGCTG CATCGACACA GGGAGGCCGC TATGATATGA GGAAATTGCT
GAA3442134 4234222331 1431233422 4111234422 13122TGTGCG
ATAACCA

108

45 (2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 50 (ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "RSERVII"

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
TGTCGATGCA GCATGGAA

18

60 (2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: other nucleic acid

- (ix) FEATURE:
(A) NAME/ KEY: misc-feature:

22

(B) OTHER INFORMATION: /desc = "FSERIX"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 21-62
5 (D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
10 GTCCAAACAT GGTTTAAGCC 4322432213 4322221223 2313114441 80
1232441213 33TCAGGGTT TCTACGGGCA
(2) INFORMATION FOR SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS:
15 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
20 (ix) FEATURE:
(A) NAME/ KEY: misc-feature
(B) OTHER INFORMATION: /desc = "RSERIX"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
25 GGCTTAAACC ATGTTGGAC 20
(2) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
30 (A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature
35 (B) OTHER INFORMATION: /desc = "Primer 1B"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
CGATTGCTGA CGCTGTTATT TGCG 24
(2) INFORMATION FOR SEQ ID NO: 27:
40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature
45 (B) OTHER INFORMATION: /desc = "Primer #63"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:
50 CTATCTTGAA ACATAAATTG AAACCA 25
(2) INFORMATION FOR SEQ ID NO: 28:
(i) SEQUENCE CHARACTERISTICS:
55 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
60 (ix) FEATURE:
(A) NAME/ KEY: misc-feature
(B) OTHER INFORMATION: /desc = "forward Primer1"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:
gacctgcagt caggcaacta 20
65 (2) INFORMATION FOR SEQ ID NO: 29:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid
 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "reverse primer 1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
 10 tagagtcgac ctgcaggcat 20

(2) INFORMATION FOR SEQ ID NO: 30:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 15 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (ix) FEATURE:
 20 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "forward primer 2"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
 gacctgcagt caggcaacta 20

25 (2) INFORMATION FOR SEQ ID NO: 31:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 (B) OTHER INFORMATION: /desc = "reverse primer 2"
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
 tagagtcgac ctgcaggcat 20

(2) INFORMATION FOR SEQ ID NO: 32:
 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2084 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
 (iii) Organism: *Bacillus amyloliquefaciens*
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 343..1794
 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTTGATGACT GATGATTGCG 60
 CTGAAGAAGT GGATCGATTG TTTGAGAAAA GAAGAAAGACC ATAAAAAATAC CTTGTCTGTC 120
 55 ATCAGACAGG GTATTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAT AAGGAATAAA 180
 GGGGGGTTGT TATTATTTA CTGATATGTA AAATATAATT TGTATAAGAA AATGAGAGGG 240
 60 AGAGGAAACA TGATTCAAAA ACGAAAGCGG ACAGTTCGT TCAGACTTGT GCTTATGTGC 300
 ACGCTGTTAT TTGTCAGTTT GCCGATTACA AAAACATCAG CC GTA AAT GGC ACG 354
 CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG 402
 65 AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA TCG GAT ATC GGA ATC ACT 450

GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA TCC GAT AAC	498
GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA	546
5 GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG	594
ATC GGC TCA CTG CAT TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT	642
10 TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA GAA GAT GTA ACT GCC GTC	690
GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG GAA TAT CAA	738
ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC	786
15 AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT	834
GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA	882
20 GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC GGC AAC TAT GAC TAT TTA	930
ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT GAT GTC GTG GCA GAG ACA	978
AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC	1026
25 CGT ATT GAT GCC GCC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG	1074
GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG	1122
GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC GAA AAC TAC TTG AAT AAA	1170
30 ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT TTC AAT TTA	1218
CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG CGT TTG CTG	1266
35 GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT	1314
GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA	1362
ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT ATT TTG ACA AGA GAA TCC	1410
40 GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA AAA GGG ACA	1458
TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA	1506
45 AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC	1554
CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC	1602
AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC GGA CCC GGC GGA TCA AAG	1650
50 CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG TAT GAC ATA	1698
ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA	1746
55 GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA	1794
GGTAATAAAA AAACACCTCC AAGCTGAGTG CGGGTATCAG CTTGGAGGTG CGTTTATTT	1854
TTCAGCCGTA TGACAAGGTC GGCATCAGGT GTGACAAATA CGGTATGCTG GCTGTCATAG	1914
60 GTGACAAATC CGGGTTTGC GCCGTTGGC TTTTCACAT GTCTGATTT TGTATAATCA	1974
ACAGGCACGG AGCCGGAATC TTTCGCCTTG GAAAAATAAG CGCGATCGT AGCTGCTTCC	2034
65 AATATGGATT GTTCATCGGG ATCGCTGCTT TTAATCACAA CGTGGGATCC	2084

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

Attorney's Docket Number:
5709.200-U.S.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

α-amylase variants

The specification of which (check only one item below):

- [] is attached hereto
[X] was filed as United States application

Application No. To Be Assigned

on November 16, 1999

and was amended

on _____

[] was filed as PCT international application

Number _____

on _____

and was amended under PCT Article 19

on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by an amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 of any provisional or foreign application(s) for patent or inventor's certificate or of any PCT international applications(s) for patent or inventor's certificate or of any PCT international applications(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR U.S. PROVISIONAL/FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicated "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

Attorney's Docket Number:

5709.200-U.S.

I hereby claim the benefit under Title 35, United States Code '120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this applications is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, '112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, '1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT
UNDER 35 U.S.C. 120:**

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	Patented	Pending	Abandoned
09/193,068	November 16, 1998	X		
PCT APPLICATIONS DESIGNATING THE U.S.				
APPLICATION NO	FILING DATE	US SERIAL NUMBERS ASSIGNED (if any)		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Steve T. Zelson Elias J. Lambiris Valeta A. Gregg Carol E. Rozek Robert L. Starnes Reza Green, Reg. No. 30,335 Reg. No. 33,728 Reg. No. 35,127 Reg. No. 36,993 Reg. No. 41,324 Reg. No. 38,475

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
 (Includes Reference to PCT International Applications)

Attorney's Docket Number:

5709.200-U.S.

5	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country
6	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country
7	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country
8	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country
9	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

Signature of Inventor 1	Signature of Inventor 2	Signature of Inventor 3
Date	Date	Date
Signature of Inventor 4	Signature of Inventor 5	Signature of Inventor 6
Date	Date	Date
Signature of Inventor 7	Signature of Inventor 8	Signature of Inventor 9
Date	Date	Date